POST-TRANSLATIONAL MODIFICATIONS OF THE EPITHELIAL-MESENCHYMAL TRANSITION TRANSCRIPTION FACTOR TWIST1: O-GLCNACYLATION AND TRANSAMIDATION

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Abstract

O-GlcNAc transferase (OGT) and transglutaminase II (TG2) are two enzyme which may promote cancer progression and metastasis in a variety of cancers. O-GlcNAcylation and transamidation are two post-translational modifications catalyzed by OGT and TG2, respectively. Epithelial-mesenchymal transition (EMT) is a complex developmental program that enables carcinoma cells to suppress their epithelial features changing to mesenchymal features. EMT has critical functions under normal physiological conditions, but many types of cancer cells take advantage of EMT to promote cancer development and metastasis. Evidence shows that OGT and TG2 may promote cancer metastasis through EMT transcription factors (EMT-TFs) but the molecular mechanisms have not been well characterized. In this study, we use OGT and TG2 overexpression H358 and Myc-CaP cell models to analyze cell phenotypic changes. We show that overexpression of TG2 or OGT can promote cell migration, and that co-overexpression of TG2 or OGT with Twist1 can promote cell migration even further. We then use immunoprecipitation to identity that Twist1 may be transamidated by TG2 and suggesting a casual molecular event for the increased cell migration. We could not show Twist1 was be O-GlcNAcylated by OGT despite co-overexpression of OGT with Twist1 promoting cell migration. These data suggest a novel pathway for Twist1 regulation and promotion of metastatic behaviors that deserves further study.

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Background

Overview of Epithelial-Mesenchymal Transition

The idea of epithelial-mesenchymal transition (EMT) arose in the 1980s from Elizabeth Hay and others when they observed that epithelial cells can lose their epithelial phenotypes and acquire mesenchymal phenotypes under certain conditions such as during development. This epithelial to mesenchymal change was first dubbed epithelial-mesenchymal transformation.(1) This concept challenged an older idea that adult epithelial cells were terminally differentiated *in vivo*. This phenomenon subsequently was named epithelial-mesenchymal transition to reflect that the phenotypic changes were reversible, and that cells could also revert from a mesenchymal state to a more epithelial cell state, termed mesenchymal-epithelial transition (MET).

Epithelial cells from different tissues have common generalized distinct features and functions. Epithelial cells display apical-basal polarity and are connected to a basal membrane. Epithelial cells themselves are connected on their lateral surfaces by tight junctions, adherens junctions and desmosomes. These junctions also function in cell-cell communication and can help epithelial cells maintain their polarity. Mesenchymal cells, on the other hand, do not have a defined cell polarity, cell-cell junctions and are dispersed in the extracellular matrix. Unlike epithelial cells, mesenchymal cells usually separate from each other (2) (Figure 1).

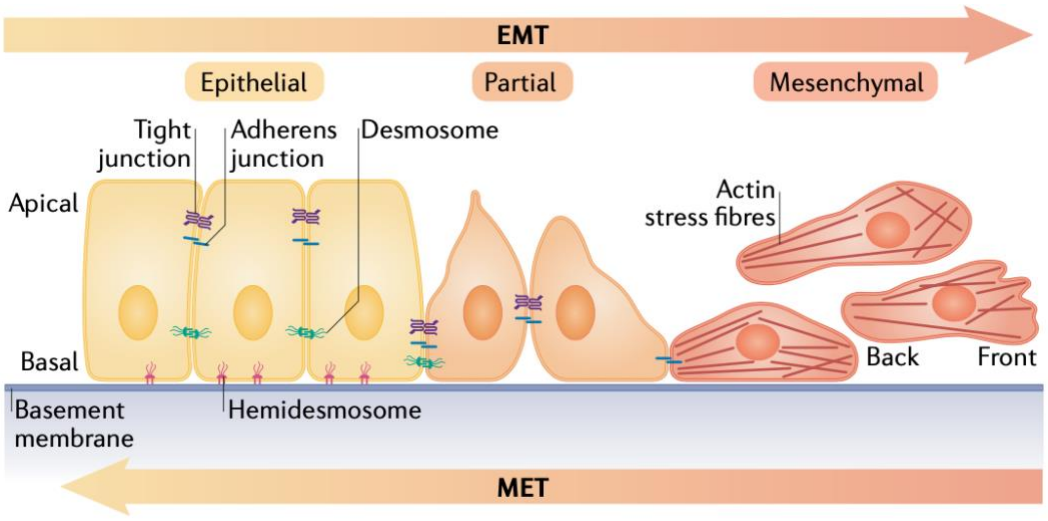


Figure 1. Outline of a typical EMT program

(taken from *New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer*. Nat Rev Mol Cell Biol, 2019). Epithelial cells displaying apical–basal polarity are held together by tight junctions, adherens junctions and desmosomes and are tethered to the underlying basement membrane by hemidesmosomes. These cells express molecules that are associated with the epithelial state and help maintain cell polarity (listed in the yellow and light orange boxes, respectively). Expression of EMT-inducing transcription factors (EMT-TFs) such as ZEB, SNAIL and TWIST inhibit the expression of genes associated with the epithelial state (listed in yellow part of figure) and concomitantly activate the expression of genes associated with the mesenchymal state (listed in the dark orange part of figure) that lead to epithelial–mesenchymal transition (EMT).

To initiate and complete the cell state transition between an epithelial and mesenchymal phenotype, a number of molecular processes are engaged. This includes the change of expression and activation level of several transcription factors, specific cell surface proteins, reorganization and expression of cytoskeletal proteins. These involved molecules have been used as biomarkers to indicate the stage of the cells in the process of epithelial-mesenchymal transition (2) (Figure 2).

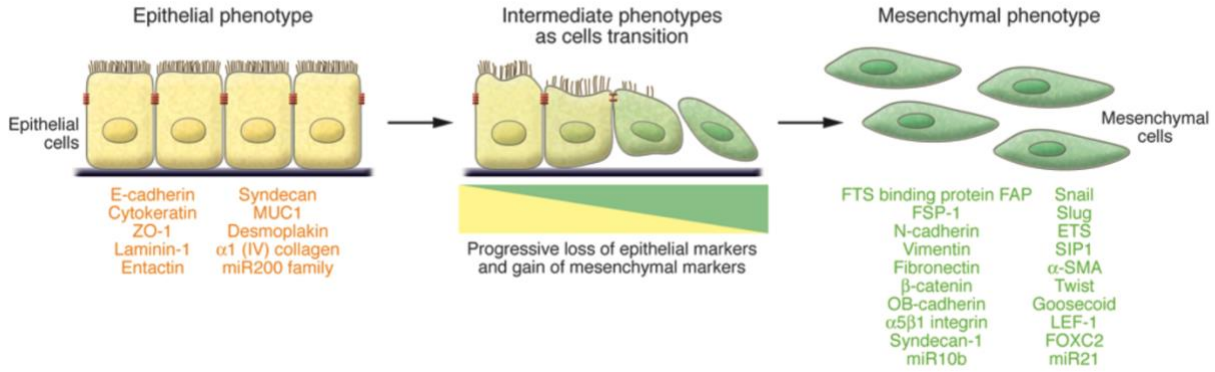


Figure 2. The basics of epithelial-mesenchymal transition

(Taken from *The basics of epithelial-mesenchymal transition*. J Clin Invest, 2009). An EMT involves a functional transition of polarized epithelial cells into mobile and ECM component–secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. Detection of cells expressing both sets of markers makes it impossible to identify all mesenchymal cells that originate from the epithelia via EMT, as many mesenchymal cells likely shed all epithelial markers once a transition is completed. For this reason, most studies in mice use irreversible epithelial cell–lineage tagging to address the full range of EMT-induced changes. ZO-1, zona occludens 1; MUC1, mucin 1, cell surface associated; miR200, microRNA 200; SIP1, survival of motor neuron protein interacting protein 1; FOXC2, forkhead box C2.

Three major subtypes of epithelial-mesenchymal transition have been characterized based on their pathophysiologic context. The first type of EMT is associated with implantation, embryo formation, and organ development, this subtype of EMT can generate mesenchymal cells which have the potential to undergo MET to form secondary epithelia. The second type of EMT is associated with wound healing, tissue regeneration, and organ fibrosis, this type of EMT is often triggered by tissue injury or inflammation, cells in this EMT are also likely to continue to exhibit epithelial-specific morphology and molecular markers, such as cytokeratin and E-cadherin, but show concomitant expression of mesenchymal markers, which is known as “partial EMT”. One example are keratinocytes which localize around a wound at the border and recapitulate part of the EMT process. They appear to acquire an intermediate phenotype known as the “metastable” state,

which allows them to move while maintaining loose contacts rather than migrating as individual cells (3). The third type of EMT occur in carcinoma cells which have genetic changes, such as overexpression of critical transcription factors (4); and epigenetic changes, such as *CDH1* promoter methylation (5), while also exhibiting oncogene and tumor suppressor gene aberrations (6). By exploiting this type of EMT, cancer cells facilitate dissociation from the primary tumor and dissemination into the blood circulation. Besides metastasis, EMT in cancer cells may also promote cancer stemness, which makes cancer cells become stem-cell like and able to differentiate into the great diversity of cell types found within the tumor mass (7). EMT is also suggested to contribute to treatment resistance in different types of cancer (8). Finally, type 3 EMT has also been shown to contribute to immune suppression and resistance to immunotherapy, by induction of regulatory T cell immunosuppression (9) or inhibit cytotoxic T lymphocyte activity (10).

Overview of Epithelial-Mesenchymal Transition Transcription Factors

The epithelial-mesenchymal transition gene expression program is initiated and mediated by several transcription factors which regulate cell-cell connection, cell polarity and motility. These transcription factors can repress key genes associated with the epithelial phenotype and upregulate mesenchymal gene expression. There are three major EMT transcription factor families: the Snail family, Twist family, and Zeb family.

The Snail transcription factor family are comprised of zinc-finger-binding transcription factors. It has three members: SNAI1, SNAI2, and SNAI3. SNAI1 and SNAI2 are well studied and have essential roles in all types of EMT, SNAI3 has also been shown to have functions in promoting tumor progression (type 3 EMT) (11) but is not essential for embryogenesis (type 1 EMT) (12).

The Snail family is best known as transcriptional repressors. Both SNAI1 and SNAI2 bind directly to the *CDH1*, or E-cadherin, gene promoter at E-box sequences to inhibit its expression (13). SNAI1 and SNAI2 also downregulates the expression of many other target genes in EMT including genes encoding for claudins, occludins, PALS1 and PATJ (14). SNAI1 has an important function to repress the expression of genes that regulate cell polarity and epithelial morphogenesis (15). In addition to regulation of EMT genes, the Snail family has been suggested to have the function of promoting cell survival, by blocking the cell cycle and inhibiting the apoptotic processes, with roles in the induction of a metastatic phenotype and the acquisition of cancer stem cell features (16). Snail family proteins are often up-regulated in multiple, different cancer types, including breast, ovarian and colorectal cancers, where they are associated with poor prognosis (17, 18, 19). The Snail family can also induce treatment resistance to chemotherapy in ovarian cancer cells (20). Several factors are known to induce the expression of Snail family members in a number of cell types. TGFβ1 and TGFβ2 can induce *SNAI1* expression in different cells and tissues (21) and *BMP4* induces *SNAI2* expression during neural crest development(22).

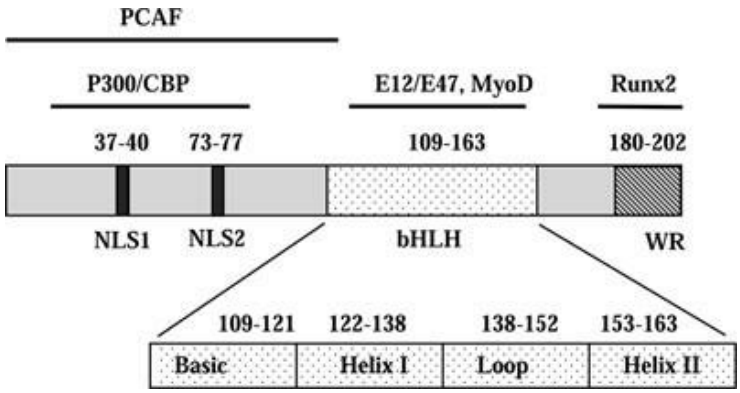


Figure 3. Structure of human Twist1

(Taken from *Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms*. Cell Res, 2012) **.** Molecular structure of the human Twist1 protein. The number of amino-acid residues for each structural domain is indicated. The regions that interact with other proteins are also indicated by solid lines. NLS1 and NLS2, nuclear localization signal sequences 1 and 2; bHLH, basic helix-loop-helix domain; WR, the tryptophan and arginine motif; CBP, cAMP-response element binding

protein (CREB)-binding protein; PCAF, p300/CBP-associated factor, Runx2, Runt-related transcription factor; E12/E47, a bHLH transcription factor that forms dimer with Twist1; MyoD, a bHLH transcription factor that regulates muscle differentiation.

The Twist Family is another major EMT transcription factor family that belongs to the basic helix-loop-helix (bHLH) superfamily of TFs and are all structurally similar (Figure 3). There are two main members of the Twist family, TWIST1 and TWIST2. TWIST1 is a 202 amino-acid residue polypeptide while TWIST2 is 160 amino-acid residues. The major functional domains of Twist proteins are the bHLH and Twist box domains and are identical between these two members (23), suggesting that TWIST1 and TWIST2 have similar biological functions. The bHLH domain, one major functional domain, serves to allow binding to E-box DNA response elements to repress or activate gene transcription (24). The Twist box or WR motif is located at the COOH-terminal of the protein, has also been postulated to have a number of important biologic functions. Evidence has shown that TWIST1 can directly bind with p53 through the Twist box and modulates key posttranslational modifications of p53 such as facilitating MDM2-mediated degradation (25). The WR motif is also required for TWIST1-induced prostate cancer metastasis, possibly through directly targeting the chromatin and gene transcription of *Hoxa9* (26). Twist family TFs are not expressed or expressed at extremely low level after embryogenesis, but can be upregulated during type 2 and 3 EMT (27). This role of Twist TFs during tumor development and progression suggests their potential to become selective targets for cancer treatment. The physiological function of Twist proteins include type 1 EMT during embryonic morphogenesis and type 2 EMT during wound healing and tissue fibrosis (28). Twist TFs regulate EMT by downregulation of important epithelial markers such as E-cadherin and by increasing the expression of the mesenchymal markers such as fibronectin, N-cadherin and vimentin. These changes lead to the reduction of cell adhesion and the promotion of cellular motility (29). Twist TFs can also induce

the expression of Snail family proteins such as SNAI2, which further enforce gene expression programs resulting in repression of epithelial genes. Some evidence has demonstrated that Twist TFs may partly induce suppression of epithelial markers such as E-cadherin indirectly by the activation of *SNAI2* (30). TWIST1 is also known for upregulating several target genes important for cancer progression. TWIST1 upregulates *Akt2* expression in breast cancer cells, which enhances cell migration, invasion and resistance to chemotherapy (31). TWIST1 also directly upregulates *BMI1*, which functions to maintain stem cell self-renewal and is frequently overexpressed in human cancers (32).

The expression of TWIST protein family members is regulated by multiple signaling pathways. The expression of TWIST1 is triggered by diverse signaling pathways linked to inflammatory-related cytokines such as TNFα and NF-κB. As shown by knockout of NF-κB *p65* in mouse fibroblasts, TWIST1 induction by TNFα can be rescued.(33) Signal transducer and activator of transcription 3 (STAT3) can also directly bind to the second proximal STAT3-binding site on the human *TWIST1* promoter and activate gene transcription. STAT3 also induces Twist family member expression at the mRNA and protein levels as demonstrated by STAT3 inhibition (34). Stress conditions also induce TWIST1 protein expression via WNT/β-catenin and hypoxia-inducible factor-1 s(HIF-1). In mouse mammary epithelial cells, TWIST1 protein expression level is associated with WNT1 levels. Using TWIST-LUC promoter reporter assays, β-catenin can dramatically activate Twist-LUC activity, confirming *TWIST1* can be activated through the WNT/β-catenin pathway (35). In human breast cancer cell lines, HIF-1α shows direct correlation with TWIST1 expression. This correlation has been confirmed *in vivo* for mouse cells overexpressing HIF-1α. Mouse cells with a mutant hypoxia response element (HRE) in the *Twist1*

promoter can prevent Twist1 overexpression induced by HIF-1α, suggesting HIF-1α upregulates *Twist1* expression by binding directly to the HRE in the *Twist1* promoter (36).

The Zeb family is another EMT TF family that includes ZEB1 (or TCF8/ δEF1) and ZEB2 (or SIP1). Zeb proteins belong to the zinc finger class of transcription factors. Their structure contains C2H2-type zinc fingers, a common DNA-binding motif that binds to CAGGTA/G E-box-like elements in the promoters of target genes.

Similar to the other EMT TF families, the physiological function of Zeb family members includes roles during embryogenesis and tissue differentiation. Zeb proteins have major functions as transcriptional suppressors by binding to E-box elements in the promoters of target genes. Conditional ZEB1 expression in epithelial cells results in the specific loss of E-cadherin expression and strongly correlates with the loss of cell aggregation and with induction of invasion *in vitro*.(37) This has been demonstrated for Zeb2 using luciferase reporter assays for many genes coding for crucial proteins involved in tight junctions, desmosomes and gap junctions such as *CLDN4*, *ZO-3* and *CDH1*.(38) Apart from direct transcriptional repressor function, Zeb family members are also known to induce the expression of many mesenchymal markers such as vimentin and N-cadherin. (39) This induction of mesenchymal proteins may be the outcome of the crosstalk between other different EMT TFs.

Overview of Post-Translational Modifications

Posttranslational modification (PTM) of proteins happens typically after protein biosynthesis, it refers to reversible or irreversible chemical changes to the protein. By definition, posttranslational

modifications happen after DNA has been transcribed into RNA and then translated into polypeptide chains, and the modification is made on the amino acid of the polypeptide chain. These chemical alterations range from the enzymatic cleavage of peptide bonds to the covalent additions of particular chemical groups, lipids, carbohydrates, or even entire proteins to amino acid side chains. These modifications change structures and properties of polypeptide chain, and consequently, the structure and function of proteins. Because of the diverse possibilities of these changes, posttranslational modifications are considered as one important way to increase the genome coding capacity. Many reversible posttranslational modifications, such as phosphorylation, are rapid and are an easy way to change protein properties. Posttranslational modifications can happen at any time after the protein has been translated with some proteins modified before folding and others modified after translocation.

There are wide variety of posttranslational modifications and they are mostly catalyzed by special enzymes that recognize specific target sequences in specific proteins. Based on the change of the protein structure and property, posttranslational modifications can be divided into several different types, the most common ones are specific cleavage of precursor proteins, formation of disulfide bonds, or covalent addition or removal of low molecular weight groups such as acetylation, methylation, phosphorylation, glycosylation, and our specific research interests, transamidation and O-GlcNAcylation. These additional chemical groups mainly change the structure and the nucleophilic properties of the target protein and have influence on protein-protein interaction, thus affect protein activity.

Transglutaminase 2 (TG2)

Transglutaminase 2 (TG2) is a member of large transglutaminase protein family. Transglutaminases are enzymes that in nature primarily catalyze atransa midation reaction, which result in the addition of the small biological amine on γ-carboxamide groups of glutamine residue side chains or formation of an isopeptide bond with the primary amine group, with subsequent release of ammonia. In many cases, ε-amino groups of lysine residue side chains can serve as an substrate and be covalently bound to the glutamine residue of the protein. TG2 is the most widely expressed and most studied member of this family. In addition to its transamidating activity, it has been shown to bind and hydrolyze GTP and ATP. Although ATP/ADP binding has no effect on transamidating activity, the calcium and GTP/GDP binding activity can determine the transamidating activity of TG2. TG2 is only active as a transglutaminase when bound to calcium and inactive when bound to GTP/GDP(40). Recent studies show TG2 is truly a multifunctional protein as it may serve as a protein disulfide isomerase, a protein kinase, and protein scaffold (41,42,43).

The major function of TG2, transamidation, takes two steps. At the first step, the sulfur of the active site cysteine (C277) performs a nucleophilic attack on the γ-carbon of the peptide bound glutamine side chain (which is the acyl-donor) and forms a thioester bond between C277 and the substrate. An ammonia molecule is released at this step as a byproduct. At the second step, the thioester bond is attacked by a primary amine or water (which is the acyl-acceptor). If the attacking group is a primary amine (either a small biological amine or the ε-group of a peptide bound lysine), the reaction is called transamidation; if it is a water molecule it is called deamination (44). The addition of a small biological amine can be considered as a post-translational modification and it

changes the properties of the substrate proteins. The formation of isopeptide bonds gives the target protein resistance to chemical and physical disruptive forces, which may stabilize the protein. Evidence has shown TG2 and other transglutaminases have functional significance in stabilizing the extracellular matrix and in preventing the release of intracellular contents of apoptotic cells to the extracellular milieu (45).

Considering the multifunctional property of TG2 and its key roles to regulate different cell activities such as cell survival, differentiation, growth, migration, invasion, and apoptosis, it won't be much of a surprise to see correlation between TG2 and tumor progression. Indeed, evidence shows that TG2 expression level is increased in different types of cancer including breast cancer (46), ovarian cancer (47), pancreatic cancer (48), lung cancer (49), prostate cancer and liver cancer (50). TG2 regulates tumor growth and cancer metastasis in different mechanisms that include cell apoptosis, promoting angiogenesis, cell invasion and migration. In renal cell carcinoma, TG2 expression promotes cell survival through crosslinking of p53 in autophagosomes and subsequent p53 depletion (51). It has been demonstrated that inhibition of TG2 expression and transamidation activity leads to inhibition of angiogenesis (52), in colorectal cancer, where TG2 regulates angiogenesis and apoptosis via the Wnt/β-catenin pathway (53). In prostate cancer cells, increase of another transglutaminase, TG4, is associated with significantly increased cell motility (54). The increased expression of TG2 in breast cancer cells contributes to their increased survival, invasion and motility, and downregulation of endogenous TG2 by siRNA inhibited fibronectin-mediated cell attachment, survival and invasion (55). In a study of pulmonary fibrosis, TG2 is shown to have the ability to mediate epithelial-mesenchymal transition via the Akt pathway, and blocking TG2 attenuates bleomycin-induced pulmonary fibrosis in mice through inhibiting EMT (56) This result

indicated the role that TG2 may play in cancer metastasis as EMT is an critical mechanism for tumor progression and transition.

O-GlcNAc transferase (OGT) and O-GlcNAcylation

UDP-N-acetylglucosamine peptide N-acetylglucosaminyl transferase, or O-GlcNAc transferase (OGT), catalyzes the transfer of a single N-acetylglucosamine from UDP-GlcNAc to a serine or threonine residue in cytoplasmic and nuclear proteins. This modification with a beta-linked N-acetylglucosamine (O-GlcNAc) is termed O-GlcNAcylation. The reverse reaction, which remove the GlcNAc moiety from proteins, is catalyzed by the enzyme O-GlcNAcase (OGA). Unlike the multiple proteins that can catalyze transamidation reactions, OGT and OGA are the only two members to regulate protein O-GlcNAcylation.

The sugar nucleotide donor for O-GlcNAcylation, Uridine-diphosphate N-acetylglucosamine (UDP-GlcNAc), is synthesized as the final product of the hexosamine biosynthetic pathway (HBP). HBP is a bypass pathway of glycolysis, with approximately 2%–5% of all cellular glucose funneled into the HBP. Glutamine-fructose-6-phosphate aminotransferase (GFAT), which converts fructose-6-phosphate to glucosamine-6-phosphate, is the first and rate-limiting enzyme of HBP (57). The HBP has an essential role in sustaining vital cellular processes. Evidence show glucose metabolism through HBP is required to sustain sufficient growth factor signaling and glutamine uptake to support cell growth & survival. Additionally, the HBP has been shown to regulate ER stress pathways to maintain protein homeostasis, enhance degradation of misfolded proteins and reduce pro-apoptotic signaling (61). Considering the correlation between glycolysis and O-GlcNAcylation, many studies have focused on revealing the role OGT plays in metabolic

disorders such as diabetes. Increasing HBP has potential involvement in the development of insulin resistance as has been shown in primary cultured adipocytes by using a GFAT inhibitor (57). Overexpression of OGT in muscle and adipose tissues resulted in the Type 2 diabetic phenotype, suggesting that O-GlcNAcylation may play an important role in the development of insulin resistance (58). Moreover, several important proteins in the insulin signaling pathway were shown to be O-GlcNAcylated, including insulin receptor substrate (IRS) proteins IRS1 and IRS2, phosphoinositide-dependent kinase 1 (PDK1), phosphatidylinositol-3-OH kinase (PI3K), and protein kinase B (AKT) (57), suggesting that O-GlcNAcylation is the link between the HBP and insulin resistance, and suggests O-GlcNAcylation may have a critical role in the development of diabetes. Besides diabetes, OGT and HBP have been shown to involved in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (59). O-GlcNAcylation can also affect cardiovascular diseases as the cardiovascular system is uniquely sensitive to metabolic changes. Recent studies indicate that O-GlcNAcylation might serve as a key player in the primary pathophysiology of some cardiovascular diseases (60).

With greater understanding of how tumor and cancer cells hijack and manipulate metabolic pathways, the relationship between O-GlcNAcylation and cancers is getting more attention. Cancer cells rewire their metabolism and signaling networks to promote growth, survival, proliferation, and rapidly proliferating cancer cells tend to produce ATP via glycolysis rather than the mitochondrial oxidative phosphorylation, even in the presence of ample oxygen. Considering the vital function of HBP, many cancer cells experience elevated flux through the HBP. In a transgenic mouse model, oncogenic RAS increases flux through glycolysis and the HBP, resulting in increased O-GlcNAcylation (62). The aberrant expression of OGT and OGA is often associated

with many different types of cancer. For example, studies demonstrate that breast cancer cell lines and patient samples contain elevated levels of OGT and O-GlcNAcylation compared to normal counterparts (63,64). Clinical study also show OGT mRNA expression is elevated in prostate cancer tissue compared to normal adjacent tissue in over 200 patients and increased mRNA was associated with a decreased patient five year survival rate (65).

Although much evidence show the correlation between O-GlcNAcylation and tumor progression, little is known about the mechanisms by which cancer cells upregulate OGT. In breast cancer cell lines, the PI3K-mTOR-MYC signaling pathway is required for elevation of OGT and O-GlcNAcylation. In the transgenic oncogene driven mouse model using MMTV-MYC derived mammary tumor epithelial cells, increased O-GlcNAcylation and OGT protein expression is associated with the tumor development from normal tissue to late carcinoma (66). In another study using a murine model of T-cell acute lymphoblastic leukemia, OGT is required for malignant transformation and c-Myc is a key controller of T cell O-GlcNAcylation. These evidence suggest Myc may play a critical role in upregulating OGT and O-GlcNAcylation in cancer cells.

The biological functions of O-GlcNAcylation are very diverse since hundreds of protein substrates can be recognized by OGT and modified. O-GlcNAcylation levels may be maintained in an optimal range by OGT and OGA at the transcriptional and post-translational levels. Maintenance of O-GlcNAc homeostasis is essential for optimal cellular function, and disruption of cellular O-GlcNAcylation may contribute to the pathogenesis of various human diseases including cancer. O-GlcNAcylation can promote cancer in many different mechanistic ways. The modulation of cellular O-GlcNAc levels has been linked to altered cellular development, mitotic progression,

growth, and survival patterns. In breast cancer cell lines which express high levels of OGT and O-GlcNAcylation, using RNAi or a pharmacological inhibition method to normalize OGT and O-GlcNAc levels anchorage-independent growth *in vitro* is blocked. Similar inhibition blunts *in vivo* primary tumor growth of breast cancer cells in transgenic mouse models (63). In prostate cancer cell lines, inhibition of OGT leads to decreased cell cycle progression and DNA replication in an MYC-dependent manner. It has been previously demonstrated that the oncoprotein c-MYC is O-GlcNAcylated at threonine 58 and this modification functions to stimulate the cell growth (67). OGT and O-GlcNAcylation can also promote cancer cell invasion and metastasis through modulation of the epithelial-mesenchymal transition (EMT). Inhibition of OGT reduces breast and prostate cancer cell EMT, invasion and metastasis *in vivo* (68, 65). The mechanism for OGT and O-GlcNAcylation promoting cancer cell EMT is often through the modification of EMT transcription factors. O-GlcNAc modification of Snail1 at serine 112 stabilizes the transcription factor and thus increases its function to attenuate E-cadherin expression and promote EMT in MCF7 breast cancer cells (69). O-GlcNAcylation also regulates cancer angiogenesis. In prostate cancer cells, reducing levels of OGT and O-GlcNAcylation blocked expression of vascular endothelial factor (VEGF) and importantly reduced angiogenic potential of prostate cancer cells *in vitro* (65). Not surprisingly, OGT and O-GlcNAcylation also modulate the glycolysis pathway, which make it more suitable for cancer cell metabolism. Many glycolytic enzymes have been shown to be direct substrates for OGT. One such enzyme is phosphofructokinase-1 (PFK-1), which catalyzes the committed step of glycolysis, the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate. PFK-1 modification by O-GlcNAc was shown to enhance glycolytic activity and pentose phosphate pathway flux, conferring a selective survival advantage to lung cancer cells (70).

Post-Translational Modifications and Epithelial-Mesenchymal Transition

During EMT, many types of posttranslational modifications (PTMs), including phosphorylation, SUMOylation, glycosylation and ubiquitination, play important roles and modulate many EMT factors.

The phosphorylation of EMT-TFs at certain amino acid residues often leads to the stabilization of the protein. For example, Phosphorylation at Thr203, Tyr342 and Ser11 stabilize Snail1; at Ser68 and Ser42 stabilize Twist1; and, at Ser585 stabilizes Zeb1. As a consequence, upregulated kinases can promote cancer cell EMT and cell invasion. The reason for phosphorylation stabilizing EMT-TFs is often because this modification blocks further possible modification for PTMs that lead to protein degradation such as ubiquitination Phosphorylation at Ser42, Thr121, and Ser123 can lead to the degradation of Twist1 . Phosphorylation can also lead to nuclear translocation. Phosphorylation at Ser82 and Ser246 is reported to induce the nuclear accumulation of Snail1.

SUMOylation is reported to happen to the Snail and Zeb families. SUMOylation at Lys116 can stabilize Snail2; while at Lys391 or Lys866 can inhibit the transcriptional activity of Zeb2.

Ubiquitination is another well-studied PTM of EMT-TFs, with the main biologic consequence resulting in protein degradation. Ubiquitination at Ser11, Lys98, Lys137 or Lys 146 leads to Snail protein degradation. Ubiquitination at Thr121, Ser123 or the C-terminal Twist box can lead to Twist1 degradation.

The glycosylation of EMT-TFs is a relatively new research topic. Understanding how cancer cells change glucose metabolism to promote tumor growth and cancer invasion and metastasis is important. O-GlcNAcylation is shown to have the ability to potentially modulate EMT. Direct evidence show Snail1 is O-GlcNAcylated at Ser112 is stabilized (69). Other studies also suggest increased O-GlcNAcylation may promote cancer cell EMT in *vivo* and *vitro* (71). The O-GlcNAcylation of other EMT-TFs remains to be discovered. Considering the complex regulation of O-GlcNAcylation and EMT and their equally complex interplay , we propose the hypothesis that Twist1 can be O-GlcNAcylated by OGT to promote the EMT. In addition, in colorectal cancer cells, the upregulation of tissue transglutaminase can promote EMT and lead to increased cell migration and invasion (72). In ovarian cancer, TGF-β-induced TG2 regulates EMT, formation of spheroids and enhances ovarian tumor metastasis by inducing EMT and a cancer stem cell phenotype (73). Though TG2 has been shown in many studies to have the ability to promote EMT and cancer metastasis, a specific molecular mechanism has not yet been discovered. For this thesis, we hypothesize that TG2 modulates EMT and the phenotype of cancer cells through direct PTM of EMT-TFs such as Twist1.

Materials and Methods

Plasmids, Antibodies, and Reagents

pRP-Hygro-CMV-hOGT, pRP-Hygro-CMV-hTGM2, pRP-Hygro-CMV-EGFP, pRP-Puro-CMV-FLAG/hTWIST1 are purchased from VectorBuilder.

Plasmid was extracted and purified using Midi Plasmid extraction kit(Qiagen).

Recombinant Anti-Transglutaminase 2 antibody (ab109200, AbCam)

Anti-O-GlcNAcylation Transferase antibody (#24083, Cell Signaling)

Recombinant Anti-DDDDK tag antibody (ab205606, AbCam)

Recombinant Anti-Vimentin antibody (ab92547, AbCam)

Anti-N Cadherin antibody (ab18203, AbCam)

Anti-E-cadherin Antibody (MABT26, Millipore)

Anti-twist Antibody (sc-81417, Santa Cruz)

Anti-Actin Antibody (sc-8432, Santa Cruz)

Goat anti-Mouse IgG (H+L) Secondary Antibody HRP-conjugated (Invitrogen)

Goat anti-Rabbit IgG (H+L) Secondary Antibody HRP-conjugated (Invitrogen)

Cell Line and Culture Conditions

Previous work in the lab generated Myc-CaP mouse prostate cancer cell line with stable Twist1 overexpression and it's control cell line and Myc-CaP mouse prostate cancer cell line with stable FLAG-Twist1 overexpression and it's control cell line, and H358 human non small cell lung cancer cell line with stable overexpression of Twist1 and it's control cell line.

Myc-CaP cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM)(Invitrogen) with 4.5 g/L D-glucose and L-glutamine, H358 cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 Medium (Invitrogen), All media were supplemented with 10% fetal bovine serum(FBS), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells were maintained at 37°C in a humidified incubator with 5% CO2 and were routinely tested for mycoplasma using the standard PCR method.

Western Blot Analysis

Cells are grown to 80% confluency before making the cell lysis. Cell lysates were prepared in RIPA buffer (Santa Cruz Biotechnology) supplemented with protease inhibitor cocktail (Roche). Equal amounts of proteins were fractionated by 10% SDS-PAGE and transferred to PVDF membrane (Bio- Rad).

Plasmid Preparation and Cell Transfection

Constructed *E.coli* with plasmid construction was inoculated in to 100 ml LB broth(Gibco) with 100ug/ml ampicillin(Sigma), after proliferating for 24h, plasmid was extract and purified using Plasmid Midiprep kit(Qiagen), use Nanodrop ONE(Thermofisher) to determine the DNA concentration and store in 4 degree fridge.

At the day prior of transfection, seed cells into 6 well plates at density of 2x10^5 cells/ml and wait cells to be 70–90% confluent at transfection. After the cells are ready, prepare 19ul Lipofectamine 3000(Thermofisher) and 750ul Opti-MEM medium(Gibco) and mix well using vortex in an EP tube. Prepare master mix of DNA by diluting 5ug/well DNA in 250 ul OptiMEM Medium, then

add 10ul P3000 reagent and mix well by pipetting. Add Lipofectamine 3000 medium to the DNA master mix and incubate for 10min, during incubation change cell culture medium to 1% fetal bovine serum medium. After incubation, add 250ul DNA master mix to each well of cell culture dropwise. After 24h, aspirate medium with transfection reagent and wash cells 2 times in PBS, change the cell culture medium to complete medium and incubation for 24h. After 24h, add selection antibiotic(hygromycin or puromycin) at the concentration determined by the kill curve experiments and select for 4-6 days. Before use cells for analyze change cell culture medium to complete medium for at least 24h.

Antibiotic Kill Curve

Seed Myc-CaP cells or H358 cells in 24-well plates, grow until reach 50% confluency. Replace the medium with fresh medium containing varying concentrations of selection antibiotic(Hygromycin 100ug/ml – 600ug/ml, Puromycin 1ug/ml – 5ug/ml). Maintain each concentration in triplicates in 24 well plate, replace the medium with antibiotic every 48 hours. Culture the cells for 7 – 10 days regularly observing the cells under light microscope. On day 10, determine the cell viability in each well either by MTT assay or by accurate cell counter. Choose a concentration of antibiotic that completely blocks the growth of cells. Use this concentration in all transfection experiments to select for resistant, transfected cells.

Wound-Healing Migration Assay

Two-dimensional migration assay was performed using a wound-healing model. Cells were grown in six-well plates for 24-48 hours to confluence. Starve cells with FBS-free medium for 24 hours to reduce cell proliferation. Multiple scratch wounds were created using a P-200 micropipette tip,

and cells were fed with fresh complete media. Two representative fields of the wound from each well were marked and images were taken at 0 and 24 hours after wounding. Relative wound closure is calculated from the ration of the initial wound area to the remaining wound area using ImageJ software.

Twist1 induction experiments involve doxycycline pre-treatment. Medium with 1ug/ml Doxycycline was added when seeding the cells. Medium with doxycycline was used for all after procedure to keep Twist1 overexpression level.

Transwell Assay

Transwell assay was performed using Corning BioCoat control inserts with 8.0 μm PET membrane. Starve cells for 24 hours to reduce cell proliferation. Before seeding the cells, fill each well of 24-well plates with 500ul complete medium, put inserts in the wells and pre-moisture the inside of inserts with serum-free medium for 5 minutes. Aspirate the inside serum-free medium, seed equal numbers of cells with serum-free medium into the inserts and incubate at 37°C and 5% CO2 for 20–24 hours. Fix cells by adding paraformaldehyde (PFA) at final concentration 4%, Wash transwell inserts at both sides of the membrane with PBS to remove debris. Incubate the bottom of inserts with 1% crystal violet in 2% ethanol for 10 minutes to stain the cells. Wash twice in water to remove exceed crystal violet. Capture several images in order to cover up all the bottom surface of the transwell insert membrane, the number of cells counted using these images will be the total number of cells in migration assay.

Immunoprecipitation

H358 and Myc-CaP cells transfected with TG2 were seeded in 10cm plates and grown into confluency. BP was added to the culture medium at 0.5 mM and incubate for 24h. Cells were lysed by RIPA buffer added with protease inhibitors. DNA was disrupted by sonication. Samples were incubated overnight at 4°C with streptavidin–agarose beads(Cytiva). After three washes with 2 M urea buffer and three washes with phosphate-buffered saline (PBS), proteins precipitated on the beads were eluted using Laemmli's buffer containing dithiothreitol (Sigma) boiled 5 min at 95°C. Samples were then analyzed by Western blot.

Dynabeads Co-IP kit(Thermofisher) was used for the immunoprecipitation of FLAG-Twist1. Dynabeads were washed and prepared based on kit protocol. Beads were conjugated with Anti-FLAG antibody(Abcam) by incubating with the antibody overnight at 37°C. After transfection and selection, H358 and Myc-CaP cells was seeded in to 10cm plates and growth into confluency. Cells were lysed by detergent reagent provided by the kit. Incubate the antibody-coated beads with cell lysis at RT for 30min, wash beads for 3 times using PBS, proteins precipitated on the beads were eluted using elution buffer provided by the kit. The concentration of protein precipitated were determined by Bicinchoninic acid assay(BCA) and then analyzed by Western blot.

Statistics

The data from transwell and scratch migration assay was performed using 2-tailed, unpaired Student's t test.

Results

Chapter I (OGT-Twist1-EMT axis)

H358 and Myc-CaP cell lines can express high levels of OGT and FLAG-Twist1 after transient transfection

To better understand the functional and phenotypic consequences of different cancer cells following enforced high expression levels of OGT, Twist, and co-overexpression of Twist1 with OGT, we used the transfection reagent Lipofectamine 3000 to transiently transfect different plasmid expression constructs. The H358 cell line is human non-small cell lung cancer cell line and Myc-CaP is s mouse prostate cancer cell line. We also used a previously generated Myc-CaP cell line with stable FLAG-tagged Twist1 overexpression (Myc-CaP FLAG-Twist1) and the isogenic control cell line with stable overexpression of an empty vector (Myc-CaP Vector). The stable cell line expressed FLAG-Twist1 as verified by Western blot (Figure 4B & 4C). For the hTwist1 plasmid construct, we also used a FLAG-tagged version. As a control we used EGFP plasmid as a visible marker to check transfection efficiency.

Before using transfected cells for further downstream experiments, we checked the expression level of green fluorescence protein (GFP)_as a visual marker detectable under the microscope. The expression levels of EGFP reached greater than 60% of cells (H358 and Myc-CaP) (Figure 1A). After transient transfection and selection, we use Western blot to check the expression level of FLAG-Twist1 and OGT in cells. In Myc-CaP stable cell lines, cells transfected with OGT plasmids showed higher expression levels of OGT protein than cells transfected with EGFP plasmid (Figure 4B). In the H358 cell line, the OGT levels were also increased in cells transfect with OGT plasmids. Also, H358 cells transfected with FLAG-Twist1 showed high expression levels of FLAG-Twist1

by Western blotting, while the H358 cells only transfected with EGFP showed no expression of Twist1 protein (Figure 4B). Based on the GFP marker levels and the Western blot results, the Lipofectamine3000 resulted in high transient transfection efficiency. These transfected cells were felt to be suitable for further immunoprecipitation and phenotypic analysis.

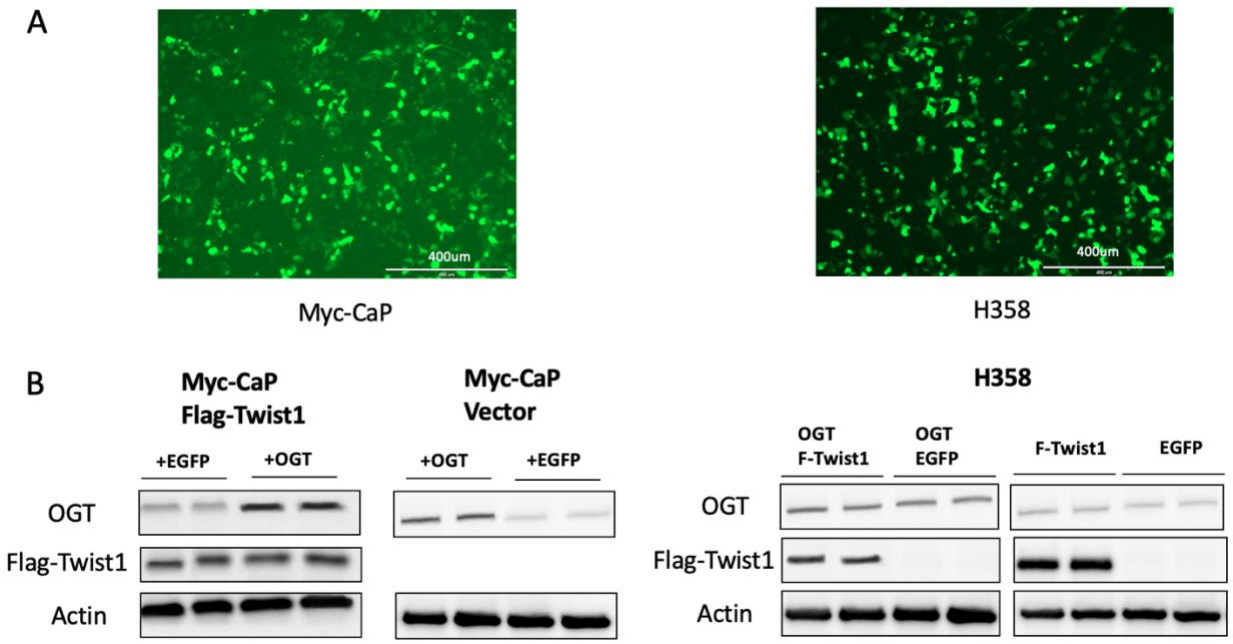


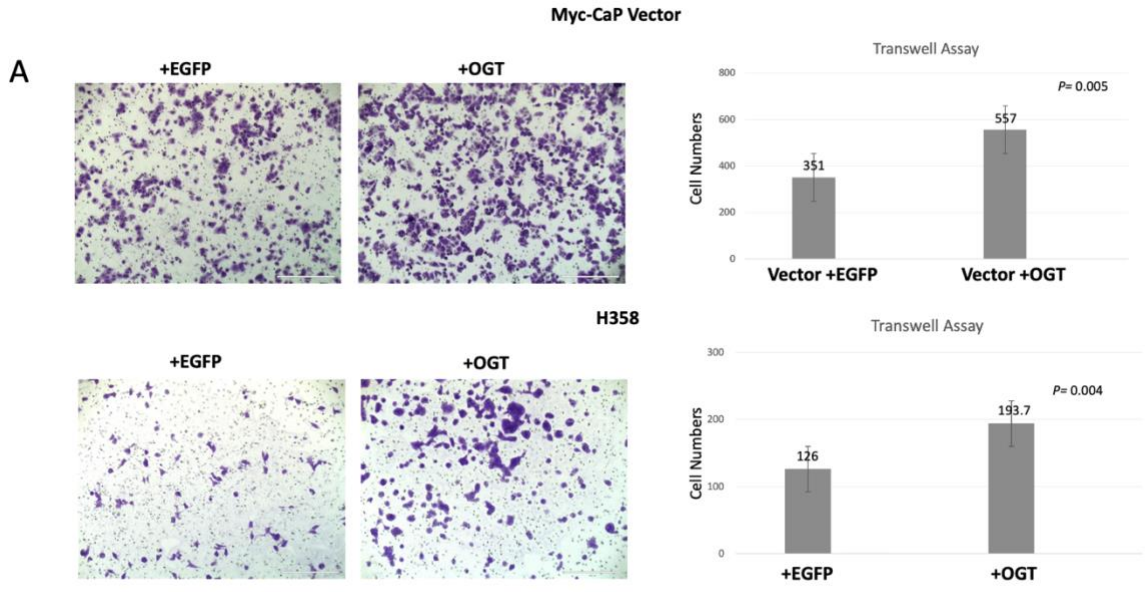
Figure 4. H358 and Myc-CaP cell lines can express high levels of target protein after transient transfections

(A) Green fluorescence marker in Myc-CaP and H358 cell lines after transfection and selection. (B) The expression level of OGT and FLAG-Twist1 in the Myc-CaP FLAG-Twist1 stable cell line was elevated as compared to the vector control cells. The expression level of OGT and FLAG-Twist1 in H358 cells transfected with OGT+FLAG-Twist1 or OGT+EGFP or only FLAG-Twist1 or only EGFP, each group has two replicates.

Overexpression of OGT can promote cell migration in Myc-CaP and H358 cells

During the EMT cancer cells will undergo several phenotypic changes including increased cell migration. The transwell and scratch assays are two different ways to measure cell migration. Myc-CaP cells are suitable for testing cell migration using both assays while H358 cells are not suitable for the scratch assay as H358 cells are hard to grow to confluency. To test our hypothesis

regarding an OGT-Twist1-EMT axis, we first made OGT overexpression Myc-CaP and H358 cell lines to see the effect of only overexpressing OGT itself in cells. After overexpressing OGT, both Myc-CaP cells and H358 cells showed increased migration ability. Myc-CaP cells transfected with EGFP had an average of 351 cells that made it through the transwell. After overexpression of OGT, the number increased ~1.6 fold to 557 cells, P=0.005(Figure 5A). For H358 cells the migration ability also increased by 1.5 fold, following OGT overexpression; cells that went through the well increased from 126 in the EGFP control to 193.7 in the OGT overexpression group, P=0.004. The scratch assay also showed similar results for Myc-CaP cells. Myc-CaP cells with OGT overexpression showed a relative migration of 1.596 compared to 1.239 with control vector, P<0.05(Figure 5B). These results are consistent with other studies showing O-GlcNAcylation may increase cancer metastasis and increased O-GlcNAcylation levels in can lead to increased cancer cell migration and invasion.



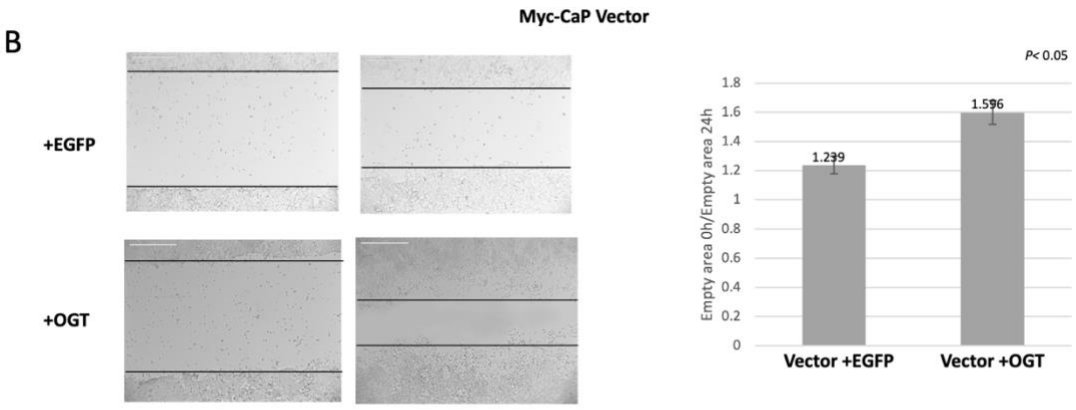


Figure 5. Overexpression of OGT can promote cell migration in Myc-CaP and H358 cells

Myc-CaP and H358 cells transfected with OGT showed increased migration by (A) transwell assay; and, (B) scratch assay, Pairwise comparisons by t-test.

Co-overexpression of OGT and Twist1 promotes H358 and Myc-CaP cells migration further

Twist1 is a well-known EMT transcription factor whose functions include promotion of EMT leading to cell migration and invasion. We used H358 and Myc-CaP FLAG-Twist1 stable cell lines to test whether overexpression of OGT with Twist1 could promote cell migration further.

The Myc-CaP cells with stable FLAG-Twist1 overexpression showed more cell migration with the transwell assay than isogenic vector control cells. We observed an increase from 351 cells to 520 cells, or by ~1.5 fold, P=0.009(Figure 6A). H358 cells transfected with FLAG-Twist1 also showed more cell migration than H358 cells transfected with EGFP; an increase from 126 cells to 225 cells, or by 1.8 fold, P=0.004(Figure 6A). The scratch assay for Myc-CaP cells also showed that Twist1 overexpression resulted in more cell migration than in the EGFP control group; the EGFP cells has a relative migration of 1.239 and the FLAG-Twist1 cells were 1.602, P<0.05. To test the functional consequences of OGT and Twist1 co-overexpression on cell migration, we made

H358 and Myc-CaP cells that co-overexpression both OGT and Twist1. Myc-CaP cells overexpressing OGT and FLAG-Twist1 showed higher cell migration ability than the control cell line and FALG-Twist1 alone cell lines. The OGT+FLAG-Twist1 Myc-CaP group averaged 807 cells that went through the well after 24-h incubation versus the control group and Twist1 group only having 351 and 520 cells, respectively. This equates to an increased ~2.3 and ~1.6 fold cell migration compared to control and FLAG-Twist1 alone groups, P=0.002 (Figure 6A). H358 cells transiently transfected with OGT+ FLAG-Twist1 also showed significant increases in cell migration ability with the transwell assay. OGT+ FLAG-Twist1 H358 cells averaged 487 cells that went through the well, as compared to the control group and FLAG-Twist1 alone group which only had 126 and 225 cells, respectively, P=0.006(Figure 6A).

For the scratch assay, Myc-CaP cells with OGT+ FLAG-Twist1 showed a relative migration of 1.790 compared to the control cells and FLAG-Twist1 alone cells that showed 1.239 and 1.602, respectively, P<0.05(Figure 6B). Based on this evidence, overexpression of either Twist1 or OGT can promote increased cell migration in H358 and Myc-CaP cells. Co-overexpression of Twist1 and OGT together can further increase cell migration. The increase in cell migration by OGT and Twist1 individually has been well studied, but the reason for the further increased migration after co-overexpression of OGT and Twist1 together is still not clear. To explore this further, we designed immunoprecipitation experiments to test the hypothesis that OGT may interact with Twist1 and result in PTM of Twist1 by OGT via an OGT-Twist1 axis.

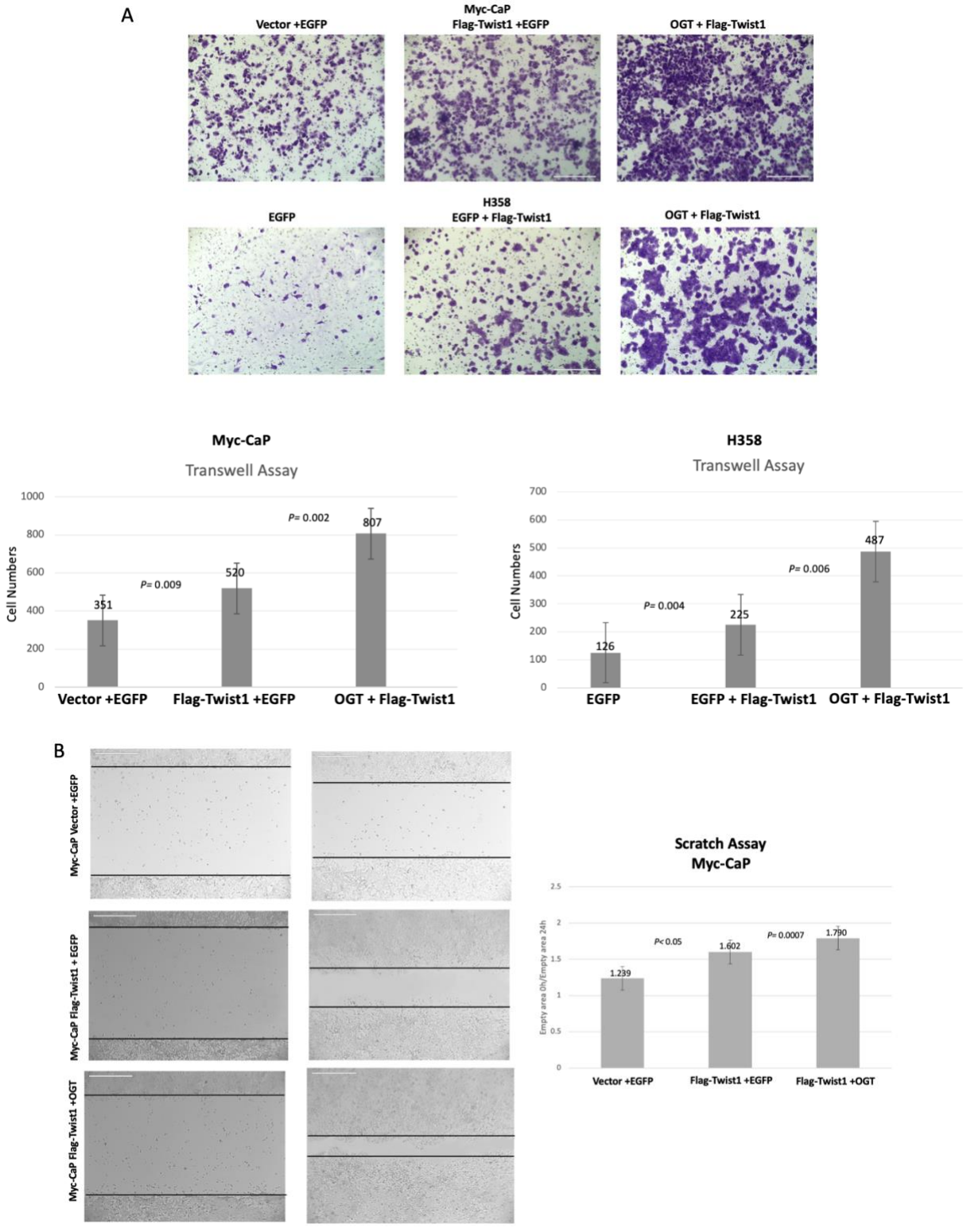


Figure 6. Overexpression of OGT and Twist1 together promotes H358 and Myc-CaP cell migration

(A) Transwell assays for Myc-CaP and H358 cells transfected with EGFP, FLAG-Twist1, and FLAG-Twist1+OGT. FLAG-Twist1 promoted cell migration in both cell types and is increased further by co-expression of OGT. Each group are triplicated and

quantification showed in bar graph. (B) Scratch assay for Myc-CaP Vector cells transfected with EGFP, Myc-CaP FLAG-Twist1 cells transfected with EGFP, and Myc-CaP FLAG-Twist1 cells transfected with OGT. FLAG-Twist1 promoted cell migration that is increased further by co-expression of OGT. Each group are triplicated and quantification showed in bar graph.

Overexpression of OGT does not lead to O-GlcNAcylation of Twist1

We transiently transfected H358 cells with both OGT and FLAG-Twist1. We also transiently transfected Myc-CaP cell with stable expression of FLAG-Twist1 with OGT. Both these cell lines were confirmed for expression of target proteins by Western blot (Figure 4). We then Western blotted for overall O-GlcNAcylation levels in both H358 and Myc-CaP cells (Figure 7A & B, lane 1). Western blotting for O-GlcNAcylation showed a smear indicating all the cellular proteins that were O-GlcNAcylated. Cell lysates were then used for FLAG immunoprecipitation pull down with magnetic agarose beads. In lanes 2 the Western blot showed that the FLAG-Twist1 protein was pulled down successfully. When then we tried to detect O-GlcNAcylation of this FLAG immunoprecipitated fraction using the same antibody as for Lane 1, but we did not detect any signal for either H358 (Figure 7A) or Myc-CaP cells (Figure 7B). Based on these results, Twist1 may not be modified by OGT or the level of O-GlcNAcylated Twist1 is too low for the reagents used.

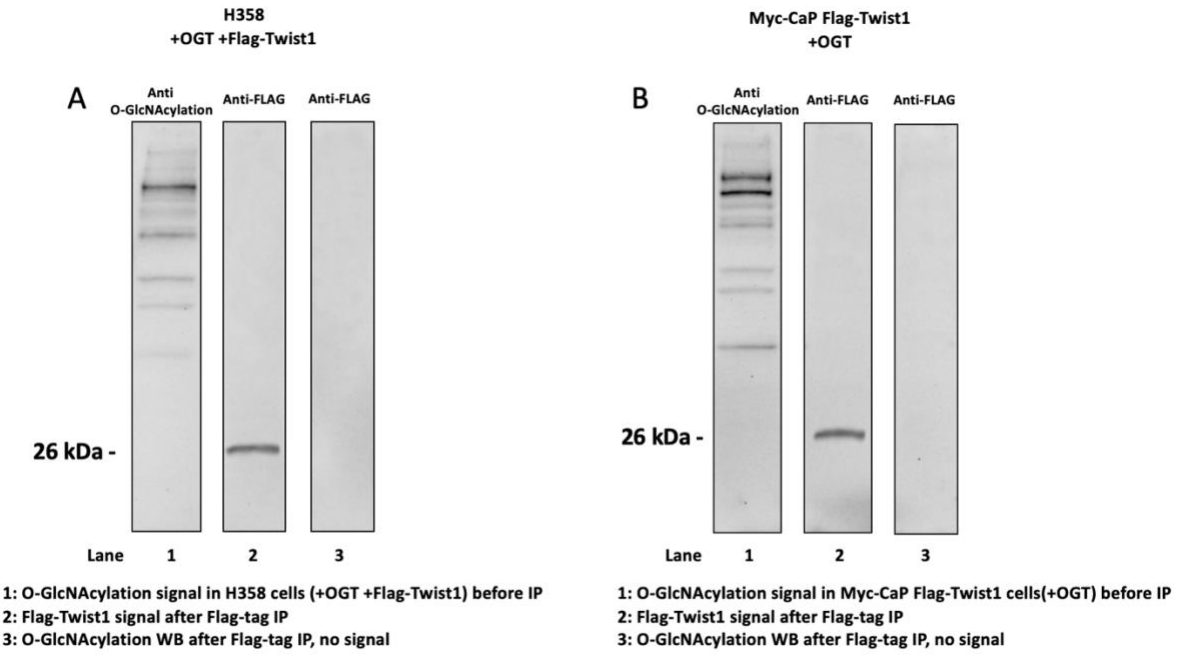


Figure 7. OGT overexpressing cells do not demonstrate O-GlcNAcylation of Twist1 by immunoprecipitation

(A) Lane 1: Western blot for O-GlcNAcylation in H358 cells transfected with OGT and FLAG-Twist1 whole cell lysate before immunoprecipitation. Lane 2: Western blot for FLAG-tag in H358 cells transfected with OGT+FLAG-Twist1 after immunoprecipitation for the FLAG-tag. Lane 3: Western blot for O-GlcNAcylation in H358 cells transfected with OGT+FLAG-Twist1 after immunoprecipitation for the FLAG-tag. (B) Lane1: Western blot for O-GlcNAcylation in Myc-CaP stable FLAG-Twist1 cells transfected with OGT whole cell lysate before immunoprecipitation. Lane 2: Western blot for FLAG-tag in Myc-CaP stable FLAG-Twist1 cells transfected with OGT after immunoprecipitation for the FLAG-tag. Lane 3: Western blot for O-GlcNAcylation in Myc-CaP stable FLAG-Twist1 cells transfected with OGT after immunoprecipitation for the FLAG-tag.

Chapter II (TG2-Twist1-EMT axis)

H358 and Myc-CaP cell lines can express high levels of TG2 and FLAG-Twist1 after transient transfection

To understand the functional and phenotypic consequences of different cancer cells following enforced high expression levels of TG2, Twist, and co-overexpression of Twist1 with TG2, we also used the transfection reagent Lipofectamine 3000 to transiently transfect different plasmid expression constructs into H358 cell line and Myc-CaP FLAG-Twist1 and it's control cell line Myc-CaP Vector generated in previously.

Before using transfected cells for downstream experiments, we checked the expression level of green fluorescence protein as in the Chapter I. After transient transfection and selection, we use Western blot to check the expression level of FLAG-Twist1 and TG2 in cells. In Myc-CaP stable cell lines, cells transfected with TG2 plasmids showed higher expression levels of TG2 protein than cells transfected with EGFP plasmid(Figure 8A). In H358 cell line, the OGT levels was also increased in cells transfected with TG2 plasmids plus EGFP than only transfected with EGFP, and H358 cells transfected with FLAG-Twist1 also showed high expression levels of FLAG-Twist1, while the H358 cells only transfected with EGFP shows no expression of Twist1 protein(Figure 8A). Based on the GFP marker levels and the Western blot results, the Lipofectamine3000 result in high transient transfection efficiency for TG2 plasmids and these transfected cells were felt to be suitable for further immunoprecipitation and phenotypic analysis.

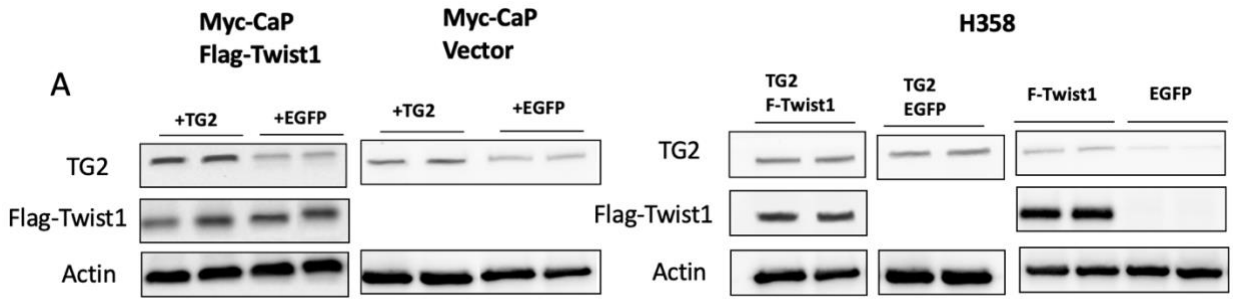


Figure 8. H358 and Myc-CaP cell lines can express high level of TG2 and FLAG-Twist1 after transient transfections

(A) The expression level of TG2 and FLAG-Twist1 in Myc-CaP FLAG-Twist1 stable cell line was elevated as compared to the vector control cells. The expression level of TG2 and FLAG-Twist1 in H358 cell line transfected with TG2+FLAG-Twist1 or TG2+EGFP or only FLAG-Twist1 or only EGFP.

Overexpression of TG2 can promote cell migration in Myc-CaP cells and H358 cells

We also use cell migration as a measurement for the cell EMT for both H358 and Myc-CaP cells. We use scratch transwell assays for Myc-CaP cells and transwell assay for H358 cells as the reason state in chapter I. To test our hypothesis regarding an TG2-Twist1-EMT axis, we first made TG2 overexpression Myc-CaP and H358 cell lines to see the effect of only overexpressing TG2 itself in cells. Like OGT, after overexpressing TG2, both Myc-CaP cells and H358 cells show increased migration ability. Myc-CaP cells transfected with EGFP had an average of 351 cells that made it through the transwell, after overexpression of TG2, the number increased ~1.7 fold to 573.3 cells, P=0.004. For H358 cells, the migration ability also increased by 1.7 fold, following TG2 overexpression; cells that went through the well increased from 126 in the EGFP control to 215.7 in the TG2 overexpression group, P=0.002(Figure 9A). For the scratch assay of the Myc-CaP cells, TG2 overexpression group showed a relative migration of 1.57, while EGFP control showed 1.239, P<0.05(Figure 9B). As a enzyme associated with cancer stem cell survival, inflammation, metastatic spread, and drug resistance, it's not surprise to find out the overexpression of TG2 can

promote cancer cell migration in these two cell lines, but whether it's trough modify EMT transcription factor Twist1 or not is yet to be determined.

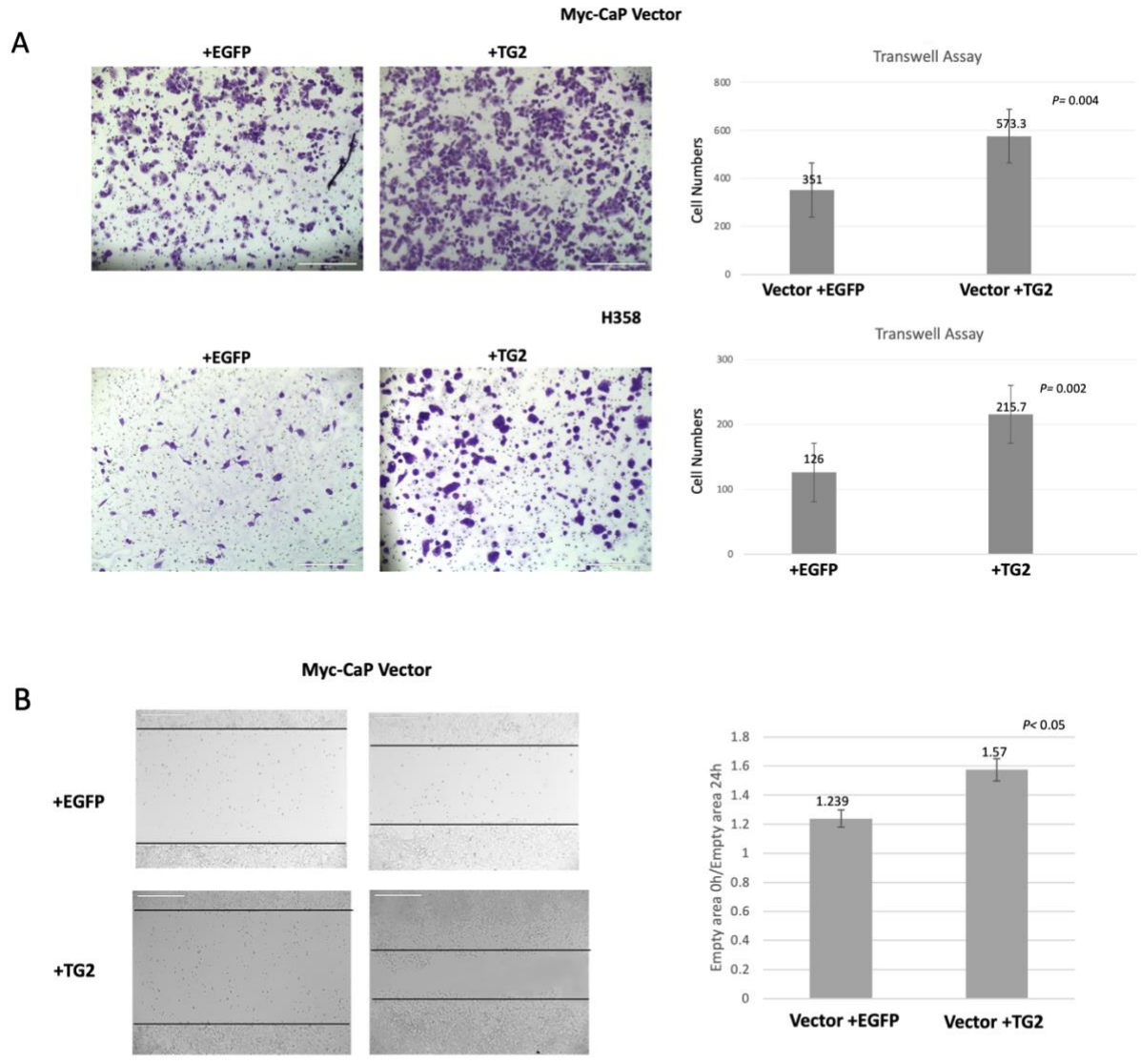


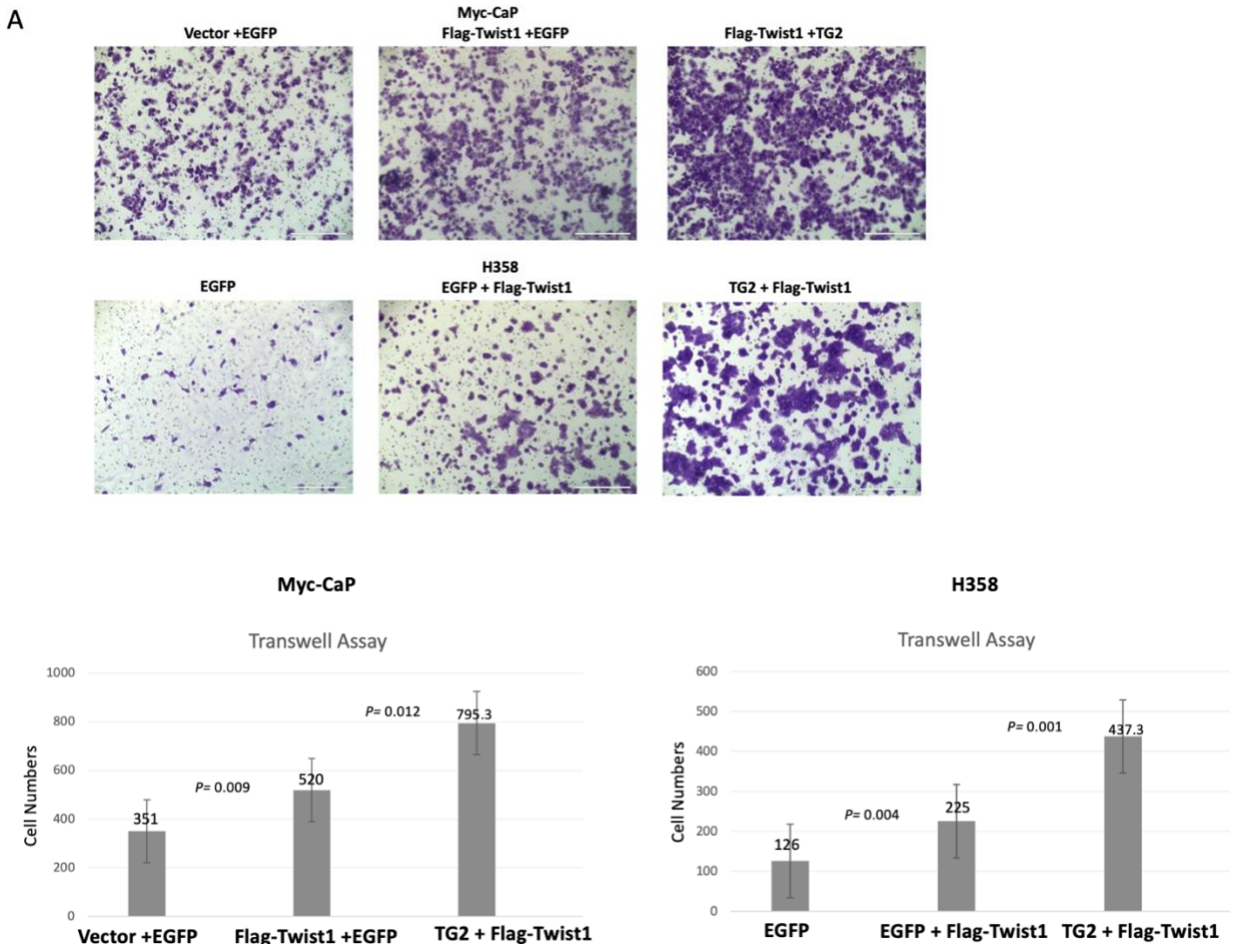
Figure 9. Overexpression of TG2 can promote cell migration in Myc-CaP cells and H358 cells

Myc-CaP vector stable cells(P=0.004) and H358 cells(P=0.002) transfected with TG2 showed increased migration in (A) transwell assay; and, (B) Scratch assay, pairwise comparisons by t-test.

Co-overexpression of TG2 and Twist1 promotes H358 and Myc-CaP cells migration further

The effect of overexpress Twist1 itself has been shown in Chapter I, the FLAG-Twist1 overexpression can promote cell migration in both H358 and Myc-CaP cells.

To test the function and relation of TG2 and Twist1, we made TG2 Twist1 co-overexpression H358 and Myc-CaP cell line. Myc-CaP cells overexpressing TG2 and FLAG-Twist1 showed higher migration ability than the control cell line and FLAG-Twist1 cell line.



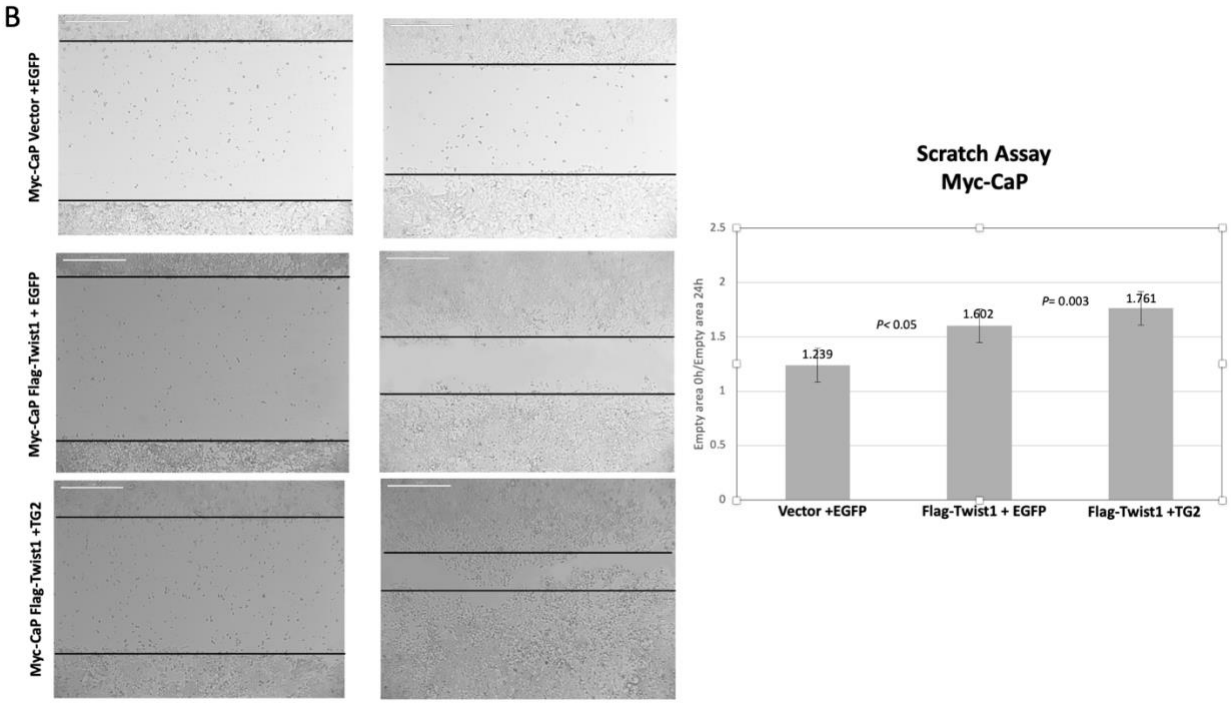


Figure 10. Overexpress TG2 and Twist1 together can promote H358 and Myc-CaP cells migration furthermore

(A) upper lane: Transwell assay for Myc-CaP Vector cells transfected with EGFP, Myc-CaP FLAG-Twist1 cells transfected with EGFP, and Myc-CaP FLAG-Twist1 cells transfected with TG2. Lower lane: Transwell assay for H358 cells transfected with EGFP, H358 cells transfected with FLAG-Twist1, and H358 cells transfected with TG2 and FLAG-Twist1. Each group are triplicated and quantification showed in bar graph. (B) Scratch assay for Myc-CaP Vector cells transfected with EGFP, Myc-CaP FLAG-Twist1 cells transfected with EGFP, and Myc-CaP FLAG-Twist1 cells transfected with TG2. Each group are triplicated and quantification showed in bar graph.

The TG2+FLAG-Twist1 Myc-CaP group has averaged 795.3 cells that went through the well after 24h incubation versus the control group and Twist1 group only having 351 and 520 cells, respectively. This equals to an increased by ~2.2 (P=0.009)and ~1.5 (P=0.012)fold compared to control and FLAG-Twist1 group(Figure 10A). H358 cells transfected with TG2+ FLAG-Twist1 also showed significant increase in migration ability in the transwell assay, the TG2+ FLAG-Twist1 group has averaged 437.3 cells that went through the well, the control group and FLAG-Twist1 group only have 126 (P=0.004)and 225 (P=0.001)cells(Figure 10A).

For the scratch assay for the Myc-CaP cells, the TG2+ FLAG-Twist1 group showed a relative migration of 1.761, the control cells and FLAG-Twist1 cells only show 1.239 and 1.602(Figure 10B). These results show overexpress either Twist1 and TG2 can promote the cell migration in H358 and Myc-CaP cells, and co-overexpression of Twist1 and TG2 can further increase cell migration.

Overexpression of TG2 leads to transamidation of Twist1

The co-overexpression of TG2 and Twist1 together could promote cell migration further suggesting the possibility of a TG2-Twist1-EMT regulation axis. To verify that Twist1 is modified by TG2 via TG2 transamidating activity, we designed the immunoprecipitation experiments to pull down transamidated proteins. Biotin-pentylamine is one primary amino source and it can outcompete other amino source inside the cell to be covalently bind with target protein through transamidation reaction. Then we can use streptavidin coated beads to pull down all the transamidated proteins and use streptavidin-HRP to detect all transamidated proteins in the cell using Western blot.

We first use streptavidin-HRP to detect the signal for all transamidated proteins in H358 cells and Myc-CaP cells co-overexpress TG2 and FLAG-Twist1 after incubating cells with biotin-pentylamine. It showed a positive smear signal on the membrane, which were the signals for all the transamidated proteins in H358 and Myc-CaP cells co-overexpressing TG2 and FLAG-Twist1 (Figure 11A and 11B, Lane 1). Then we use the streptavidin-coated beads to pull down biotinylated proteins in modified H358 and Myc-CaP cells lysate. After elution of proteins on the beads, the protein solution was then analyzed by Western blot. The pull-down is very efficient and almost all

biotinylated proteins were been precipitated in both cell lines (Figure 11A and 11B, Lane 2). We then detected FLAG-Twist1 in the pull-down solutions using anti-FLAG antibody and got positive results for both cell lines (Figure 11A and 11B, Lane 3). These results suggest Twist1 may be transamidated by the TG2 under co-overexpression condition in H358 and Myc-CaP cells and the modification may promote cancer cell migration in these cell lines.

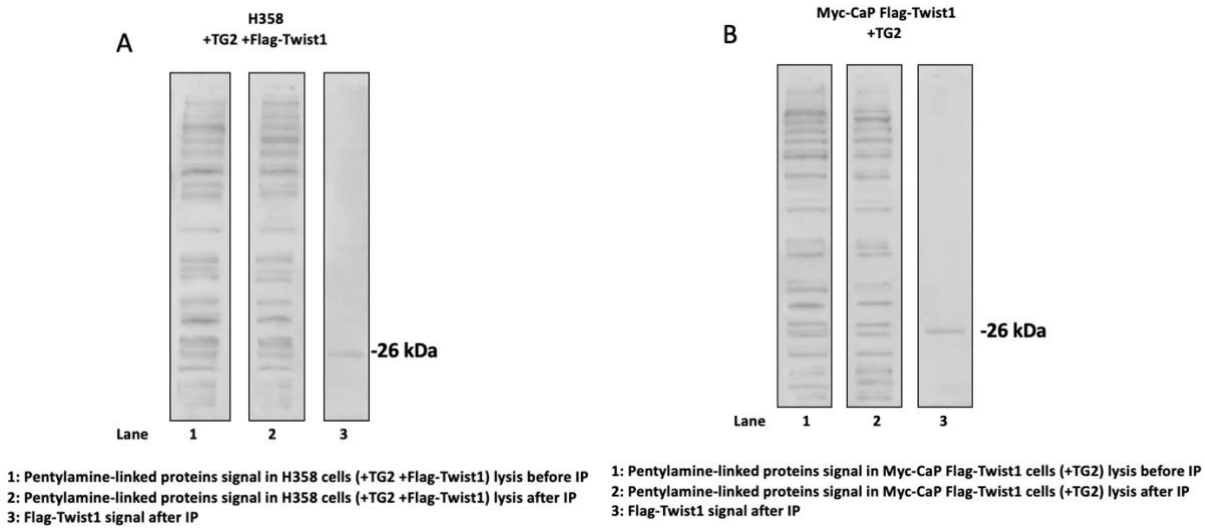


Figure 11. Twist1 is transamidated in H358 and Myc-CaP cells overexpress TG2 and Twist1

(A) Lane1: Western blot for biotinylated proteins in H358 cells transfected with TG2 and FLAG-Twist1 whole cell lysate before immunoprecipitation; Lane 2: After IP, Western blot for biotinylated proteins in immunoprecipitated proteins solution; Lane 3: Western blot for FLAG-Twist1 in immunoprecipitated proteins solution using anti-FLAG antibody. (B) Lane1: Western blot for biotinylated proteins in Myc-CaP FLAG-Twist1 stable cells transfected with TG2 whole cell lysate before immunoprecipitation; Lane 2: After IP, Western blot for biotinylated proteins in immunoprecipitated proteins solution; Lane 3: Western blot for FLAG-Twist1 in immunoprecipitated proteins solution using anti-FLAG antibody.

Bioinformatic prediction for the possible transamidation site on Twist1

Based on our immunoprecipitation results, Twist1 may be transamidated by TG2. We then used bioinformatic methods to predict the possible modification site based on TG2 preferred substrate sequences (74). A screening system to elucidate primary structures surrounding reactive glutamine residue(s) was shown to be preferred by TG enzymes. The screening system used phage clones

expressing peptides that incorporated biotin-labeled primary amines from the catalytic reactions of TG2. Based on these results, several preferred sequences are known: QxPϕD(P), QxPϕ, and QxxϕDP, where x and ϕ represent a non-conserved and hydrophobic amino acid, respectively. We then marked all the glutamines in the Twist1 protein sequence in bold (see below) and the glutamine in position 27 (Q27, marked in red) matched the pattern QxPϕ. This glutamine appears to be best candidate for where TG2 may transamidate Twist1.

MM**Q**DVSSSPVSPADDSLSNSEEEPDR**Q****Q**PPSGKRGGRKRRSSRRSAGGGAGPGGAAGGGVGGGDEPGSPA**Q**
GKRGKKSAGCGGGGGAGGGGGSSSGGGSP**Q**SYEEL**Q**T**Q**RVMANVRER**Q**RT**Q**SLNEAFAALRKIIPTLPSDKLSKI
QTLKLAARYIDFLY**Q**VL**Q**SDELDSKMASCSYVAHERLSYAFSVWRMEGAWSMSASH

Figure 12. Bioinformatic prediction for the possible transamidation site on Twist1 protein

Twist1 protein sequence, all glutamine marked in bold and the possible TG2 modified glutamine marked in red.

Discussion

In this study, we used transient overexpression of two putative cancer progression enzymes, O-GlcNAc transferase (OGT) and transglutaminase II (TG2), and showed increased functional pro-cancer functions *in* vitro. We used a human non-small cancer cell model (NSCLC) and a mouse prostate cancer cell model to construct overexpression cell lines of OGT or TG2 that showed increased cancer cell migration. In addition, co-overexpression with Twist1, a critical EMT-TF, with either OGT or TG2 resulted in at least an additive effect on cell migration. Using immunoprecipitation pull down assays with Twist1, we were also able to demonstrate a possible mechanistic link between TG2 and EMT to promote increased cell migration.

For the detection of transamidation on Twist1, we use biotin-pentylamine to incubate with cells to have all transamidated proteins biotinylated, and use streptavidin coated beads to pull down all biotinylated proteins. Then we use the Western blot to detect if there are Twist1 proteins among all the biotinylated proteins pull-down and got positive signal. This suggest that Twist1 might be modified under TG2 and Twist1 overexpression conditions in H358 and Myc-CaP cells, and this modification may have the ability to promote the EMT and increase cell migration ability. Whether Twist1 is transamidated in other cancer cell lines or under normal conditions need to be determined with further experiments. Using a similar strategy we tried to detect O-GlcNAcylation of Twist1 in OGT overexpressing cells but only obtained negative results. OGT has been reported to modify other EMT-TF such as Snail1. Further work is needed to determine whether OGT can modify and regulate Twist1 under other conditions.

Although we didn't have positive data show that Twist1 is O-GlcNAcylated, there may be other regulating pathways or crosstalk between OGT and Twist1 and EMT. Many studies have suggested there is connection between hyper-O-GlcNAcylation and EMT. In endometrial cancer cell line, the pharmacological inhibition of OGA can lead to hyper-O-GlcNAcylation, which leads to enhanced the expression of EMT-associated genes *WNT5B* and *FOXC2* (75), *WNT5B* has been identified as a key regulatory factor that governs the phenotype of cancer cells by activating canonical and non-canonical WNT signaling and has been shown to promote the overall epithelial to mesenchymal transition including many EMT-TFs, and these EMT-TFs and other EMT related genes may have functional relations with Twist1 and may promote EMT together with Twist1(76). FOXC2 transcription factor, which is involved in specifying mesenchymal cell fate during embryogenesis, is associated with the metastatic capabilities of cancer cells, and may also have functional correlations with Twist1(77).

The hexosamine biosynthetic pathway(HBP) is an important pathway for sensing metabolic status, which is one of the cancer hallmarks. The HBP requires the input of several molecules, such as glucose, glutamine, UTP, and acetate, and thus plays a key role in sensing and integrating information on nutrient availability, and many studies showed upregulation of HBP in many types of cancer(71). Twist1 was shown to have the ability to induce rate limiting enzymes in HBP(71). Increased HBP can generate more of the reaction substrate for protein O-GlcNAcylation. The increasing of HBP and OGT together may promote protein O-GlcNAcylation further more than only overexpression OGT, and the increased protein O-GlcNAcylation level may promote cancer cell migration. Another EMT-TF Snail1 can also be O-GlcNAcylated and increased OGT level

may also increase the O-GlcNAcylation of Snail1 and stabilize it(69), this may increase the crosstalk between EMT-TFs and may increase cancer cell EMT.

As an swiss-knife protein enzyme, TG2 has many other functions besides catalyzing protein transamidation. TG2 also serves as a G protein for several seven transmembrane receptors and catalyse Ca2+-dependent post-translational modifications of proteins by introducing protein-protein cross-links. Several mechanisms have been reported through which TG2 promotes cancer survival, tumor progression and invasion, many of these effects are attributed to the extracellularly located TG2. TG2, acting as a protein crosslinking enzyme, can modify the structure and stability of extracellular matrix (ECM) in a way that it supports integrin-dependent ECM binding and migration of cancer cells. Extracellular TG2 can crosslink S100A4 promoting metastasis (78). TG2 acts also as an integrin co-receptor for the β1, β3, β4, and β5 integrins, and facilitates integrin-mediated signaling pathways, which partly promote the growth factor signaling pathway, thus promote cell growth, partly activate the PI3K/AKT mediated-cell survival pathway leading to inhibition of apoptosis (79).

Apart from the transamidation of Twist1, TG2 can also modulate cancer cell EMT through other molecular pathways. TG2 expression level was shown to have correlation with many E-cadherin repressors such as Zeb1 in ovarian cancer (80). TG2 also induced the PI3K/Akt activation and GSK3β inactivation in A431 tumor cells and this increased Snail and MMP-9 expression resulting in higher cell motility. TG2 also upregulated NF-κB activity, which also enhanced Snail and MMP-9 expression resulting in greater cell motility; interestingly, this was associated with the

formation of a TG2/NF-κB complex. TG2 facilitated acquisition of a mesenchymal phenotype, which was reversed by inhibitors of PI3K, GSK3 and NF-κB (81).

Increased expression of OGT was reported in different clinical studies for different cancers, and patients whose tumors had high levels of OGT, had poor prognosis, overexpression of OGT in breast cervix and thyroid cancer cells can also increase their migration and invasion ability. Although it is not known what the key mediators of this response are, many inhibitors targeting OGT have been developed and are tested for their anti-cancer efficiency. These inhibitors includes substrate analogues, High through screen(HTS)-derived inhibitors and bisubstrate inhibitors (82). Although there is a relatively large number of compounds claimed to inhibit OGT, many of these lack specificity and even the best of them have not been vigorously tested in animal models. Two OGT inhibitors that are tested in animal models are 5SGlcNHex and OSMI-1. 5SGlcNHex is a metabolic inhibitor that is converted into its active form inside the cell and the compound decreases O-GlcNAcylation in multiple tissues (83). OSMI-1 was shown to cause a delay in tumor progression of pancreatic cancer but no delivery route and pharmacokinetic/pharmacodynamic data were reported (84). Although current results show OGT small-molecule inhibitors have shown promising antitumor effects, there are still many to concern such as off-target effect and tissue specificity problem as OGT is important enzyme for both physiological and pathological conditions.

Compare with OGT, TG2 has more functions including protein transamidation, protein cross-linking and GTP binding. TG2 is essential for not just the stabilization of the extracellular matrix, its unregulated activity has also been implicated in celiac disease, fibrosis, and cancer metastasis,

among other disorders. The range of pathologies in which TG2 is implicated demonstrates the need for potent TG2-specific inhibitors. Many evidences show collectively that the pathogenic role of TG2 is primarily linked to its role in protein cross-linking and transamidating, with little evidence so far to suggest the involvement of its GTPase activity. However, trapping TG2 in an unnatural conformation, relative to its cellular localization, could alter its biological effects. Therefore, the design of TG2 inhibitors that alter its conformational flexibility may be important for the use of these compounds as therapeutics. By far, there are varies inhibitors targeting at different functions of TG2 developed, including irreversible inhibitor such as halomethyl carbonyl inhibitors and 3-Halo-4,5-dihydroisoxazole inhibitors; and reversible inhibitor acyl-donor substrate competitives (85). Current compounds that target the transamidase site include irreversible inhibitor NC9, VA4 and VA5, and reversible inhibitor CP4d (86). All of these inhibitor show good anticancer efficacy invitro but tissue specificity and off-target issue are still two important hinders for these inhibitors to become successful cancer treatment.

As an important part of Structural-Functional study, the specific modification site is also of great interests. We use the bioinformatic method to predict the possible modification site on Twist1 protein and find the glutamine 27 matches with the preferred substrate sequences. Besides the bioinformatic prediction, the method of detection of the PTM site of other EMT TFs has been well described in other studies. Sang Yoon et al use Mass Spectrometry method discovered the O-GlcNAcylation site on Snail1 protein, which can also be used in the detection of transamidation on Twist1. “Point mutation” method is one useful tool in structural-functional study, by substituting the modification site amino acid to other amino acid such as asparagine, the modification site can be disrupted. By expressing the mutant protein dominantly in cell model, the

functions of the Twist1 protein and the relations between TG2 and Twist1 can be further explained using other phenotypic analysis.

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